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PAC1 receptor blockade reduces central nociceptive activity: new approach for primary headache?

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Reviewer 1

Excellent manuscript. I have only some minor points.
Thank you for sharing this judgement.

1) Please use "migraine patient" instead of "migraineur"
The word migraineur is correct (OED); there is no pressing, globally accepted view to insert two words when there is one English word that can be used.

2) page 11, line 44: "Experiments were conducted all studies were conducted..." Please correct
Thanks for picking up this mistake. It has now been corrected.

3) Could you please state in which years and in which laboratory exactly the experiments were conducted
The generation of the antibody Ab181, in vitro characterization of Ab181, pharmacokinetic and pharmacodynamic analyses as well as the immunohistochemistry were all conducted between 2011 and 2013 in the Amgen laboratories in Thousand Oaks, CA, USA. The electrophysiology experiments were all conducted between 2012 and 2013 at the University of California San Francisco, CA, USA. We have now stated this in the manuscript.

4) page 24: I think it is too early to conclude that there is only a peripheral mode of action. Given the differences between the animal model and humans reagrding blood brain barrier etc., it might be that in humans there is a central site of action. Also, it is not yet proven that high concentrations of Ab181 are needed for the main mechanism. It could also be that the main mechanism is located in the CNS and that small amounts of Ab181 are sufficient.
We completely agree with this opinion. We have now amended the sentence accordingly.

Reviewer #2

The authors describe the development of a rodent-specific PAC1 receptor antibody (Ab181) and its effect on nociceptive neuronal activity in the trigemino-cervical complex. By doing so, they show that AB181 inhibits stimulus-evoked activity in the trigemino-cervical complex but not spontaneous neuronal activity. Using immunohistochemistry, they demonstrate that the antibody binds in the trigeminal ganglion and SPG but not within the CNS. The authors conclude that a PAC1 receptor antibody could provide potential benefit in treatment of primary headache. This manuscript adds important findings to the field because the potential benefit of PACAP antagonism in primary headache prevention is an ongoing discussion. However, I have few points to be addressed:

Results:

Dose response studies at 48h and 3.25 h. How did the authors choose these time points?

The time points were chosen to confirm that the receptor occupancy of Ab181 has reached and stayed at the desired pharmacodynamic measures during the entire duration of the electrophysiology experiments. We have now specified this in the manuscript.

Effect of Ab181 on neuronal activity in TCC: N number is missing. How many in vivo experiments in how many rats were performed? This is important for the interpretation of the main findings. In the methods section under the subheading general surgical preparation we had already stated that for the electrophysiological experiments we have used 24 animals. In each animal we have only recorded the electrophysiological signal from one site. We have now specified this in the manuscript.

Discussion:

Page 21 line14: The authors state that their data "provide a plausible biology for a clinical effect of PAC1 blockade". I would tone down this statement. The demonstration of inhibition of stimulated activity in the trigemino-cervical complex in an experimental animal model that might be associated with the pathophysiology of primary headaches does not justify such a conclusion.

We have now modified the sentence accordingly.

The authors state that the antagonism at the PAC1 receptor may offer a new strategy for the preventive treatment of primary headache disorders. This "new strategy" is somehow contradicted by the fact that a clinical phase-2 trial with a monoclonal PAC1 receptor antibody has already been reported to be ineffective in the preventive treatment of migraine. Instead of presenting a "new strategy" this paper supports the hypotheses that, despite the current clinical evidence, antagonism of the PAC1 receptor might offer some therapeutically gain in primary headache disorders. Accordingly, the authors discuss that PAC1 antibodies might work rather in migraine patients with autonomic symptoms or in cluster headache. I would suggest restructuring this paragraph under this aspect. Additionally, please provide the citation of the abstract of the phase 2 AMG 301 study.

We are aware of the fact that the phase 2 trial with AMG301 was negative. However, in our view it is much too early to conclude that the mechanism is ineffective in the preventive treatment of migraine. The main reason is that AMG 301 had a much lower affinity for the PAC1 receptor compared to Ab181. Given the amount of clinical and preclinical evidence supporting significant role of PACAP in the pathophysiology of migraine, it seems more likely that the lack of efficacy was based more on the specifications of AMG301 than on the mechanism itself. On top of this we have to consider the lack of distinction between migraine with and without autonomic symptoms and the complete lack of data on trigeminal autonomic cephalalgias. We will have more insight on the subject when the trials with monoclonal PACAP antibodies will have concluded and the results become available. Taken together, as we believe that AMG301 is not a good example to conclude that the mechanism is ineffective in migraine and as we have no data on trigeminoautonomic cephalalgias such as cluster headache, we have written that targeting the PAC1 receptor *may* offer a new strategy for the preventive treatment of *primary headache disorders*. Finally, we would like to cite the AMG301 data but they have only been presented as an e-poster at the EHF meeting 2019 and are not included in the abstract book published in the Journal of Headache and Pain. Hence, as much as we would like to, we can't cite the abstract.

Minor points

Introduction, line 44: The authors state that infusion of VIP "less reliably" induces migraine-like attacks. VIP does not induce migraine attacks as also demonstrated by the cited paper by Rahmann et al, Cephalalgia 2008 ("None of the subjects reported a migraine attack after VIP infusion."). Please correct.

We are aware of the work of Rahmann et al. However, the data is not that clear since Amin et al (Brain 2014; 137: 779-794) have shown that VIP may trigger attacks in some individuals. We therefore chose to use the terms "less reliably".

Results: Fig 3.2. -> Please add in the results text the referral to the designated graph (A,B,C,D)

We have now corrected this.

Abstract

Pituitary adenylate cyclase activating polypeptide-38 (PACAP38) may play an important role in primary headaches. Preclinical evidence suggests that PACAP38 modulates trigeminal nociceptive activity mainly through PAC_1 receptors while clinical studies report that plasma concentrations of PACAP38 are elevated in spontaneous attacks of cluster headache and migraine and normalize after treatment with sumatriptan. Intravenous infusion of PACAP38 induces migraine-like attacks in migraineurs and cluster-like attacks in cluster headache patients.

A rodent-specific PAC_1 receptor antibody Ab181 was developed and its effect on nociceptive neuronal activity in the trigeminocervical complex was investigated *in vivo* in an electrophysiological model relevant to primary headaches.

Ab181 is potent and selective at the rat PAC_1 receptor and provides near maximum target coverage at 10 mg/kg for more than 48 hours. Without affecting spontaneous neuronal activity, Ab181 effectively inhibits stimulus-evoked activity in the trigeminocervical complex. Immunohistochemical analysis revealed its binding in the trigeminal ganglion and sphenopalatine ganglion but not within the CNS suggesting a peripheral site of action.

The pharmacological approach using a specific PAC_1 receptor antibody could provide a novel mechanism with a potential clinical efficacy in the treatment of primary headaches.

PAC_1 receptor blockade reduces central nociceptive activity- new approach for primary headache?

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Abstract

Pituitary adenylate cyclase activating polypeptide-38 (PACAP38) may play an important role in primary headaches. Preclinical evidence suggests that PACAP38 modulates trigeminal nociceptive activity mainly through PAC_1 receptors while clinical studies report that plasma concentrations of PACAP38 are elevated in spontaneous attacks of cluster headache and migraine and normalize after treatment with sumatriptan. Intravenous infusion of PACAP38 induces migraine-like attacks in migraineurs and cluster-like attacks in cluster headache patients.

A rodent-specific PAC_1 receptor antibody Ab181 was developed and its effect on nociceptive neuronal activity in the trigeminocervical complex was investigated *in vivo* in an electrophysiological model relevant to primary headaches.

Ab181 is potent and selective at the rat PAC_1 receptor and provides near maximum target coverage at 10 mg/kg for more than 48 hours. Without affecting spontaneous neuronal activity, Ab181 effectively inhibits stimulus-evoked activity in the trigeminocervical complex. Immunohistochemical analysis revealed its binding in the trigeminal ganglion and sphenopalatine ganglion but not within the CNS suggesting a peripheral site of action.

The pharmacological approach using a specific PAC_1 receptor antibody could provide a novel mechanism with a potential clinical efficacy in the treatment of primary headaches.

Introduction

Migraine and cluster headache are highly disabling disorders [53] involving activation of the trigeminovascular system, which includes the perivascular meningeal afferents and the trigeminal ganglion in the peripheral nervous system, and the trigeminocervical complex (TCC) as well as higher brain structures including the hypothalamus, thalamus and periaqueductal gray in the central nervous system [18,34].

Pituitary adenylate cyclase activating polypeptide-38 (PACAP38) has received increasing attention in the context of migraine and cluster headache. PACAP38 is a 38-amino acid neuropeptide that is structurally and functionally related to vasoactive intestinal peptide (VIP) [27,40]. Both neuropeptides act on the same set of receptors, namely PAC_1, $VPAC_1$ and $VPAC_2$ receptors, which belong to the G-protein-coupled receptors of the secretin family [27]. PACAP38 [44] and VIP [22] have vasodilatory properties and play a major role in parasympathetic communication [7,27,36]. In cluster headache both neuropeptides are released during a spontaneous attack [15,52]. In migraine, despite some shared biology, both molecules have important differences. During a spontaneous migraine attack PACAP38 is released into the cranial circulation regardless of the presence of autonomic symptoms [51,55] while VIP is only released if cranial autonomic symptoms accompany the attack [17]. The infusion of PACAP38 [2,26,45], but less reliably VIP [43], induce migraine-like attacks in which can be effectively treated with sumatriptan. Sumatriptan normalizes elevated levels of PACAP38 during migraine [55]. These findings strikingly resemble the preclinical and clinical observations made with calcitonin gene-related peptide (CGRP) [14,16,17,37,57] which plays a prominent role in the pathophysiology of migraine and cluster headache and

has been proven to be a validated target in the treatment of both disorders [13,18,24,30,39,47].

A large body of preclinical evidence has supported and begun to dissect the mechanisms behind these clinical observations. The fact that PACAP38 and VIP share similar affinities to the $VPAC_1$ and $VPAC_2$ receptors but that the PAC_1 receptor has a 100- to 1000-fold higher affinity to PACAP38 [9] has led to the conclusion that the relevant action of PACAP38 is likely to be mediated mainly through the PAC_1 receptor [55]. These findings are supported by *in vivo* studies that demonstrate the release of PACAP38 into the cranial circulation upon peripheral trigeminal activation [55,56] and the facilitation of nociceptive neuronal transmission in the TCC that is reversible by the administration of a PAC_1 receptor antagonist [1].

These observations suggest that targeting the PAC_1 receptor may offer an effective and highly selective approach to reduce trigeminal activation and offer a novel target for the treatment of primary headaches. We therefore developed a potent and selective monoclonal mouse anti-rat PAC_1 antibody (Ab181), fully characterized the pharmacologic and pharmacokinetic properties of the agent, and studied its effect on nociceptive neuronal activity within the TCC in an *in vivo* model that has been proven to be highly predictive for clinical efficacy in primary headaches [19-21,50]. Preliminary results have been presented at the 5th European Headache and Migraine Trust International Congress [32] and the International Headache Congress [33].

Methods

The generation and *in vitro* characterization of Ab181, the pharmacodynamic and pharmacokinetic analyses as well as the immunohistochemistry were conducted between 2009 and 2013 in the Amgen laboratories in Thousand Oaks, CA, USA. The electrophysiological studies were conducted between 2012 and 2013 in the laboratory of the Headache Group at the Department of Neurology, University of California, San Francisco, CA, USA.

The generation of the Ab181

Mice anti-rat PAC_1-specific monoclonal antibodies were generated using a conventional immunization method. Five 4-6-week old hybrid 129xC57BL/6 mice (Charles River Laboratories, Hollister, CA, USA) received three rounds of immunizations with soluble rat PAC_1, chemically conjugated to Padre Peptides (Pan DR Helper T-cell epitopes). Mice were immunized SQ/IP with up to 50 µg of antigen mixed or emulsified in either Freund's complete adjuvant (Cat# 77140, Pierce, Thermo Fisher Scientific, Waltham, MA, USA), RIBI (Cat# S6322, Sigma-Aldrich, St. Louis, MO, USA) or Poly I: C/CpG. Soluble PAC_1 receptor polypeptides containing the N-terminal extracellular domains (ECDs) of rat PAC_1 (amino acids 1-135 of GenBank accession no. NM133511.1) were generated by transiently cotransfecting 293-6E cells. All mice were maintained according to the regulations of the Amgen Institutional Animal Care and Use Committees (IACUC) in Thousand Oaks, CA. Mice with the highest detected FACS titer to Rat PAC_1 expressed on CHO AMID cells were selected for fusion. Four days prior to spleen harvest, mice selected for fusion were given a final IP boost of 50 µg soluble rat PAC_1 in PBS (Cat# 14040, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA). B cell hybridomas were obtained by fusing immune splenocytes with

nonsecreting murine myeloma cells, Sp2/0-Ag14 (American Type Culture Collection), at a ratio of 2.5:1 by electrofusion. Ab181 is identified through screening assays including binding competition, functional blocking, and receptor selectivity against the rat PAC_1 receptor.

In vitro characterization of A~~A~~b181

Potency and selectivity of Ab~~A~~181 were analyzed *in vitro* cell-based functional assay.

Cell culture

In house developed stable $rPAC_1$/CHO cells were grown in Ham's F12 nutrient mixture (Cat# 11765, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA); 10% fetal bovine serum (FBS) (Cat# 10099, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA); 1X Penicillin-Streptomycin-Glutamine (Cat# 10378, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA); 400 µg/mL G418 (Cat# 10131, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA); 250 µg/ml Zeocin (Cat# R250-01, Invitrogen, Thermo Fisher Scientific Waltham, MA, USA). All cell flasks were maintained in incubators at 37°C with 5% CO_2. U2OS (Cat# HTB-96™, ATCC, Manassas, VA, USA) cells were grown in the medium of McCoy's 5A (Cat# 16600, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Cat# 10099, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA), 1x L-glutamine (Cat# 25030, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA), 1X Penicillin-Streptomycin-Glutamine and 1 X MEM Non-Essential Amino Acids (Cat# 11140, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA). All cell flasks were maintained in incubator at 37°C with 5% CO_2.

BacMam virus construct

The $rVPAC_1$ and $rVPAC_2$ BacMam virus constructs were prepared in house. The titer of $rVPAC_1$ BacMam virus is 7.01 X 10^8 IU/ml, and the titer of $rVPAC_1$ BacMam virus is 5.75 X 10^8 IU/ml.

Preparation of BacMam virus transduced $rVPAC_1$ and $rVAPC_2$ cells:

U2OS cells were cultured in T-75 flask until the cell density reach to 70 - 80% confluent before transduction. Cell medium was removed from flask and the cells were rinsed with 1 X PBS (Cat# 14040, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) once, then Versene (Cat# 15040, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) was added to detach the cells. 3 X 10^6 U2OS cells were resuspended with culture medium and mixed with $rVPAC_1$ or $rVPAC_2$ BacMam virus at a concentration of 50 multiplicity of infection (MOI)/cell. The cell mixture was further incubated overnight for assay.

Cell-based functional assay

The cAMP assay was performed by using LANCE® cAMP ultra assay kit (Cat # TRF0263, PerkinElmer Inc., Waltham, MA, USA) to determine the activity of Ab181. Assay buffer contain Ham's F12 nutrient mixture (Cat# 11765, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA), 0.1% bovine serum albumin (BSA) (Cat# CR84-100, PerkinElmer, Inc., Waltham, MA, USA), 1 mM IBMX (Cat# I5879, Sigma-Aldrich, St. Louis, MO, USA). Agonist dose response curve was first run to determine the appropriate concentration to be used in subsequent antagonism studies (data not shown) (Figure 1 and Table 1).

The antagonist activity of Ab181 was carried out by using EC_{80} of PACAP38 (Cat# H-8430, Bachem, Bubendorf, Switzerland) or Maxadilan (Cat# H-6734, Bachem, Bubendorf,

Switzerland). PACAP6-38 (Cat# H-2734, Bachem, Bubendorf, Switzerland), a PAC_1 receptor antagonist, was used as a positive control. Ab181 (0.5 pM – 1 μM) was pre-incubated with the rPAC1-CHO cell suspension (2,000 cells/well) at room temperature for 30 min prior to the addition of agonists. PACAP38 or maxadilan was then added and incubated with the mixture for additional 15 min at room temperature. The reaction was stopped by adding detection mix of Eu-cAMP tracer and ULight^{TM}-anti-cAMP (Cat# TRF0263, PerkinElmer Inc., Waltham, MA, USA) to all wells. After a 45-min incubation at room temperature, the assay plate was read on an EnVision instrument (Cat# 2105-0100, Perkin-Elmer Inc., Waltham, MA, USA).

Same assay method was used to determine the activity of Ab181 on $rVPAC_1$ and $rVPAC_2$ receptors. The activity of VIP (Cat#: H-3775, Bachem, Bubendorf, Switzerland), a $rVPAC_1$ and $rVPAC_2$ receptor agonist, was first evaluated and the EC_{80} concentration of VIP was used in the assay to determine the activity of Ab181 on these receptors.

All results were analyzed using GraphPad Prism software's nonlinear regression curve fit (GraphPad Software, Inc., La Jolla, CA, USA) and data are presented as mean ± SD. Data from the agonist dose-response curves were used to calculate the half maximal effective concentration (EC_{50}) and the half maximal inhibitory concentration (IC_{50}) values for agonist and antagonist studies, respectively.

Pharmacokinetic and pharmacodynamic analysis

Male naïve Sprague Dawley rats, 6-12 weeks from either Taconic Farms Inc. (Oxnard, CA, USA) or Harlan Laboratories (Indianapolis, IN, USA) at the average age of initiation of treatment were used and all procedures in this report were conducted in compliance with

the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare. Animals were group-housed in non-sterile, ventilated micro-isolator housing on corn cob bedding in Amgen's Assessment and Accreditation of Laboratory Animal Committee (AAALAC)-accredited facility with controlled temperature (70 ± 5º F), relative humidity (50 ± 20%), and 12-h light/dark cycles (0600 to 1800). Animals had *ad libitum* access to pelleted feed (Harlan Teklad 2020X, Indianapolis, IN, USA) and water (on-site generated reverse osmosis) via automatic watering system.

Test and control materials

Ab181 was generated from Amgen and diluted to a series of concentrations in A5Su (10 mM sodium acetate, 9% sucrose, pH = 5.0). An isotype control was used as a dummy antibody in A5Su at a concentration of 5 mg/ml. Maxadilan (trifluoroacetate salt, Bachem, Bubendorf, Switzerland) was used and a dosing solution was freshly prepared daily by dissolving maxadilan in 1X DPBS (Dulbecco's phosphate buffered saline) (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 0.5 µg/ml.

Laser Doppler Imaging

A laser Doppler imager (Moor Instruments, Ltd, Wilmington, DE, USA) was used to measure DBF on the shaved skin of the rat abdomen.

Following anesthetic with propofol on the test day, the rat's abdominal area was shaved and each animal was placed in a supine position on a temperature-controlled circulating warm-water heating pad to help maintain a stable body temperature during the study. After a 10 to 15-min stabilization period, a black rubber O-ring (0.925 cm inner diameter, O-Rings West, Seattle, WA, USA) was placed on the rat abdomen without directly positioning it over

a visible blood vessel. After placement of an O-ring on the selected area, a baseline (BL) DBF measurement was taken. After the BL scan, the maxadilan solution prepared fresh daily in 20 µl vehicle (DPBS) was injected intradermally at the center of the O-ring. The post-maxadilan DBF was measured either every 15 min over a 60-min period or at specified time points such as 15 and 30 min. The O-ring serves as an area of interest in which the DBF will be analyzed within the O-ring. Ab181 was prepared in A5Su at different concentrations depend on the dose range and given in a single bolus i.v. injection. In this report, MIIBF was measured and expressed as % change from the baseline [100 x (individual post-agonist flux-individual baseline flux)/individual baseline flux] or as % inhibition [(Mean of % change from BL from vehicle-treated animals - individual % change from BL from drug treated animals)/ Mean % change from BL from vehicle-treated animals].

Dose-response study at 48 h post Ab181 treatment

Ab181 was administered via penile vein or tail vein at various doses (0.1, 0.3, 1 and 10 mg/kg). Forty-eight h later, rats received an intradermal maxadilan injection (10 ng in 20 µl DPBS) followed by post-maxadilan DBF scans every 15 min over a 60-min period. Following the final DBF scan, three rats that had been pre-treated with 10 mg/kg of Ab181 were allowed to recover from the propofol anesthesia, returned to their home cages and underwent a second DBF measurement at 168 h post-drug administration. Serum PK samples were taken via tail vein immediately prior to the first maxadilan challenge at 48 and 168 h post Ab181 treatment. The post MIIBF scan time point for the later studies was determined based on the post-maxadilan DBF response from 15 to 60 min.

Dose-response study at 3.25 h post Ab181 treatment

Ab181 was administered via the penile vein at various doses (0.1, 0.3, 1, 3 and 10 mg/kg). Three hours later, rats received an intradermal maxadilan injection (10 ng of maxadilan in 20 µl DPBS) followed by a post-MIIBF scan 15 min later. Serum PK samples were taken via tail vein immediately after the DBF scan (i.e., 3.25 h post Ab181 treatment). The time point was chosen to confirm that the receptor occupancy of Ab181 had reached and stayed at the desired pharmacodynamic measures when performing the electrophysiological studies.

Time-course study of Ab181 at a dose of 10 mg/kg

Ab181 was dosed intravenously at 10 mg/kg via either rat tail vein or penile vein at various pre-treatment times (0.5, 3, 6.25 h) prior to maxadilan challenge. The full time-course study was comprised of several experiments, including the 48- and 168 h post-Ab181 treatment studies described above.

Statistical analysis of laser Doppler flow experiments

All DBF results were expressed as the mean ± SEM. A one-way ANOVA followed by Dunnett's Multiple Comparison Test (MCT) was used to assess the statistical significance of Ab181 effects relative to either vehicle or the control antibody. A $p<0.05$ was used to determine significance between any two groups. ED_{50} values were calculated in GraphPad Prism following logarithmic transformation and nonlinear fit of data using a sigmoidal dose-response model with variable slope and with the top constrained to the resulting mean percent change in the corresponding control group (vehicle or control antibody-treated group) and bottom constrained to zero.

Electrophysiological recordings

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Francisco. Experiments were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals, the ARRIVE guidelines and the guidelines of the Committee for Research and Ethical Issues of the International Association of the Study of Pain (IASP).

General surgical preparation

Twenty-four male Sprague-Dawley rats (Charles River Laboratories, Hollister, CA, USA) were used in the experiments. In each animal only one experiment was conducted and recordings were performed from one site. The animals were anesthetized by an induction with a single dose of pentobarbital (60 mg/kg i.p.; Nembutal, Lundbeck, Deerfield, IL, USA) followed by a continuous infusion of propofol (20-25 mg/kg/h i.v.; Propoflo, Abbott, Abbott Park, IL, USA) for maintenance throughout the entire experiment. For the administration of the anesthetic and drugs both femoral veins were cannulated. The left femoral artery was cannulated for the continuous monitoring of arterial blood pressure.

Physiological monitoring

Rats were placed on a self-regulating homeothermic blanket system with a rectal probe (Harvard Apparatus, Holliston, MA, USA) and core body temperature was maintained at 37 ± 0.5 ˚C. Arterial blood pressure was monitored from the femoral artery using a transducer (DTX Plus DT-XX, Becton Dickinson, Sandy, UT, USA) connected to an amplifier (PM-1000, CWE, Ardmore, PA, USA). Following a tracheostomy, animals were mechanically ventilated (3-5 ml/min, 75-90 strokes/min; 7025, Ugo Basile, Comerio, VA, Italy) with oxygen-enriched air and end-expiratory CO_2 was kept between 3.5 and 4.5%. Data on arterial blood pressure

and CO_2 concentration were continuously displayed and fed into a data acquisition system (Power 1401, Cambridge Electronic Design-CED, Cambridge, UK) and saved on a hard disk.

Recording preparation

The rat's heads were fixed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). A craniotomy was performed over the parietal cortex with a dental burr using constant irrigation to reduce heat production. With this procedure the middle meningeal artery (MMA) was exposed without lesioning the dura mater. For electrical stimulation a bipolar stimulating electrode (NE200, Rhodes Medical Instruments, Summerland, CA, USA) was placed above the MMA touching the dura mater at either side of the blood vessel. The electrode was connected to a stimulus isolation unit (SIU5A, Grass Instruments, Quincy, MA, USA).

For the extracellular recording of neuronal activity in the trigeminocervical complex (TCC) a C1 partial hemilaminectomy was performed and the spinal dura mater was removed. A tungsten electrode with a nominal impedance of 1 MΩ (TM31A10, World Precision Instruments, Sarasota, FL, USA) was then introduced in the TCC near the dorsal root entry zone. For the localization of optimal site for the extracellular recording, the electrode was advanced or retracted in 5 µm steps with a piezoelectric motor-driven micromanipulator. Wide dynamic range (WDR) neurons with convergent input from the dura mater and the facial skin were identified by their responsiveness to electrical stimulation of the perivascular afferents surrounding the MMA as well as innocuous brush and noxious pinch of the skin innervated by the first branch of the trigeminal nerve.

Stimulation of meningeal afferents and recording in the TCC

Electrical stimulation of the perivascular meningeal afferents was performed applying electrical square wave pulses (10-18 V, 0.1-0.2 ms, 0.5 Hz, 20 sweeps) (S88, Grass Instruments, Quincy, MA, USA).

The stimulus-evoked neuronal signal as well as the neuronal background activity were acquired by the recording electrode placed in the TCC. The electrical signal was fed into a headstage amplifier (NL100AK, Neurolog, Digitimer Welwyn Garden City, Hertfordshire, UK) and passed to an AC preamplifier (NL104, Neurolog, Digitimer Welwyn Garden City, Hertfordshire, UK) set to a gain of 1000x. The signal was then passed through a band-pass filter (bandwidth 300 Hz to 10 kHz) (NL125/126, Neurolog, Digitimer Welwyn Garden City, Hertfordshire, UK) and a 60 Hz noise eliminator (Humbug, Quest Scientific, Vancouver, BC, Canada) before further amplification by an AC-DC amplifier (NL106, Neurolog, Digitimer Welwyn Garden City, Hertfordshire, UK). This signal was fed to a gated amplitude discriminator (NL201 Neurolog, Digitimer Welwyn Garden City, Hertfordshire, UK) and a data acquisition system (Power 1401, Cambridge Electronic Design-CED, Cambridge, UK). Data was collected, analyzed and stored using Spike 5.2 software (Cambridge Electronic Design-CED, Hertfordshire, UK). The output of the gated amplitude discriminator was also fed into an audio amplifier (NL120, Neurolog, Digitimer Welwyn Garden City, Hertfordshire, UK) and loudspeaker as well as an oscilloscope to assist spike discrimination from background activity. For the analysis of stimulus-evoked neuronal activity post-stimulus histograms were produced online. Background activity gated through the amplitude discriminator was collected into successive bins.

Experimental protocol and drug administration

After completing the surgical procedure, the animals had a resting period of 30 min. Following this period, baseline recordings were obtained. These were obtained by calculating the mean of 3 series of post-stimulus histograms, each consisting of 20 electrical stimuli.

Following the assessment of the baseline values Ab181 (10 mg/kg) or its vehicle (A5Su) were administered intravenously over 1 min. Due to the pharmacological properties of the antibody the animals underwent a second resting period of 2.5 h to allow sufficient time for the antibody to bind its target. After this second resting sumatriptan (10 mg/kg) or vehicle were administered intravenously over 1 min followed by another resting period of 30 min. Post-stimulus histograms were then established 180, 185, 190, 195, 200, 205, 210 and 225 min after the administration of the first pharmacological intervention (Ab181 or vehicle) (Figure 3.1). Based on the treatments described above, animals were divided in 3 treatment groups, group 1 receiving the Ab181 (intervention 1) and sumatriptan vehicle (intervention 2), group 2 receiving vehicle (intervention 1) and sumatriptan (intervention 2) and group 3 receiving vehicle at both interventions (Figure 3.1).

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software (IBM Corporation, Armonk, NY, USA). Data are expressed as percentages of baseline values with standard errors of the mean (±SEM). Effects within a treatment group were calculated using the analysis of variance (ANOVA) with repeated measures applying the Greenhouse-Geisser correction of the assumption of sphericity was violated. Bonferroni correction was applied for multiple comparisons. Statistical significance was assumed at $p < 0.05$. For a detailed comparison of

individual data points with the baseline value within one treatment group the dependent *t*-test was used.

Immunohistochemistry

Adult male Sprague Dawley rats (n = 3 per group) were injected intravenously with Ab181 or a monoclonal mouse isotype control antibody against an unrelated target or vehicle. After 3.5 h, animals were terminally anesthesized by FatalPlus™ (Vortech Pharmaceuticals, Dearborn, MI, USA) and perfused with ice cold phosphate-buffered saline (PBS) at pH 7.4 (Cat# 14040, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) followed by 4% paraformaldehyde (Cat# P6148, Sigma-Aldrich, St. Louis, MO, USA) in PBS (Cat# 14040, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA). The SPG, TG and brains were dissected and cryoprotected with 30% sucrose in PBS. 12 µm sections were cut, washed in PBS and incubated in 3% normal goat serum (NGS) and 0.3% TritonX-100 in PBS for one hour at room temperature. Sections were then washed in PBS and incubated with AlexaFluor™488 goat anti-mouse IgG (Invitrogen, Thermo Fisher Scientific Waltham, MA, USA) for 1 h at room temperature. After final washes in PBS (Cat# 14040, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA), sections were cover slipped with Vectashield mounting medium with DAPI (Cat# H-1200, Vector Labs, Burlingame, CA, USA). The following additional controls were conducted: A set of sections from Ab181-dosed rats was incubated with secondary antibodies against rabbit (AlexaFluor™488 goat anti-rabbit IgG, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) as negative control. As positive control for PAC_1 staining a set of vehicle sections was incubated with 10 µg/ml Ab181 in blocking solution at 4 °C for 48 h before further processing with AlexaFluor™488 goat anti-mouse IgG (Invitrogen, Thermo Fisher Scientific Waltham, MA, USA) as described above.

Results

In vitro potency of the PAC_1 receptor antibody Ab181

Ab181 is a full antagonist of the rat PAC_1 receptor. It dose-dependently inhibited PAC_1 receptor agonist PACAP38 or maxadilan, a PAC_1 selective receptor agonist, induced cAMP accumulation in CHO cells expressing rat PAC_1 receptors with IC_{50} of 20 ± 3.3 nM (*n* = 3) and 4.5 ± 0.1 nM (*n* = 2), respectively (Figure 1, Table 1). Ab181 is selective to the PAC_1 receptor. IC_{50} at rat $VPAC_1$ and rat $VPAC_2$ receptors against the agonist VIP is greater than 100 nM, the highest concentration tested.

Pharmacokinetic and pharmacodynamic effect of Ab181

Dose-response study at 48 h post Ab181 treatment

Pretreatment of Ab181 48 h prior to maxadilan challenge prevented the maxadilan-induced increase in dermal blood flow (MIIDBF). The change in dermal blood flow (DBF) over 60 min post-maxadilan is depicted in Figure 2.1A. There was a statistically significant difference ($p < 0.001$) between the vehicle-treated group and each of three Ab181 dose groups (0.3, 1 and 10 mg/kg) at 15 min post maxadilan injection with a calculated ED_{50} of 0.16 mg/kg ($CI_{95\%}$ = 0.10-0.26 mg/kg). Serum concentration of Ab181 is plotted on the Y-axis of Figure 2.1B.

Dose-response study at 3.25 h post Ab181 treatment

A shorter pretreatment of Ab181 at various doses was also evaluated. A fifteen minute post-maxadilan measurement was used in this study. The change in MIIDBF after 3.25 h post-Ab181 is depicted in Figure 2.2. There was a statistically significant inhibition of the MIIDBF following dose of 1, 3 and 10 mg/kg at 3.25 h post treatment ($p < 0.01$), resulting in a

calculated ED_{50} of 0.95 mg/kg ($CI_{95\%}$ = 0.59-1.51 mg/kg). Serum concentration of Ab181 is plotted on the Y-axis of Figure 2.2.

Time-course study of Ab181 at a dose of 10 mg/kg

A fifteen minute post-maxadilan measurement was used in this study. Ab181 at 10 mg/kg produced a statistically significant inhibition of the MIIDBF starting ($p < 0.05$) from a pretreatment time of 0.75 h and lasting through the last measured 168 h time point (Figure 2.3). The resulting mean serum concentration at 1, 3, 6, 48 and 168 h post drug treatment is plotted on the Y-axis in Figure 2.3.

Electrophysiological recordings

Ab181 inhibits stimulus-evoked responses in the TCC

The intravenous administration of Ab181 (Figure 3.1) induced a long-lasting inhibition of stimulus-evoked nociceptive neuronal activity within the TCC ($F_{1.48, 5.91} = 8.43$, $p = 0.022$). Compared to the baseline value, the effect was significant at all investigated time points throughout the entire observational period. Maximum inhibition reached -43 ± 13% ($t_7 = 3.41$, $p = 0.011$) compared to baseline.

Sumatriptan, which was administered as a positive control, significantly reduced stimulus-evoked responses in the TCC ($F_{2.21, 15.49}$=4.97, $p = 0.019$). Similar to the Ab181 group, the inhibiting effect of sumatriptan was significant at all investigated time points reaching a maximum inhibition of -42 ± 15% ($t_7 = 2.78$, $p = 0.027$) compared to baseline without recovering until the end of the observational period.

In contrast, the intravenous administration of the vehicle did not affect stimulus-evoked neuronal activity ($F_{2.21, 15.48} = 2.45$, $p = 0.115$; Figure 3.2A, B).

Neuronal background activity in the TCC is not affected by Ab181

In contrast to the observed effect on stimulus-evoked neuronal activity, intravenous administration of Ab181 does not attenuate unspecific neuronal background activity within the TCC ($F_{1.32, 5.27} = 0.767$, $p = 0.456$). The same lack of effect on background activity was observed in animals treated with sumatriptan ($F_{2.33, 16.28} = 0.821$, $p = 0.474$) and those treated with vehicle ($F_{1.68, 11.78} = 0.99$, $p = 0.385$; Figure 3.2C).

Arterial blood pressure is unaffected by Ab181

Intravenous administration of Ab181 does not affect arterial blood pressure ($F_{1.69, 6.78} = 0.656$, $p = 0.525$). In contrast, sumatriptan induced a significant decrease in arterial blood pressure ($F_{2.55, 17.84} = 5.82$, $p = 0.008$) throughout the entire observational period, reaching a maximum reduction of -13 ± 3% ($t_7 = 4.89$, $p = 0.002$) compared to baseline without recovering until the end of the experiment. In the vehicle control group arterial blood pressure was unaffected ($F_{2.29, 16.02} = 0.93$, $p = 0.426$; Figure 3.2D).

Immunohistochemistry

To investigate whether Ab181 or the control antibody distributed into tissues of interest after intravenous administration, we treated satellite rats from the electrophysiology study, but dissected and fixed tissues of interest at the 3.5 h time point after injection. Using fluorescently labeled secondary antibodies against the Fc-portion of the antibodies we were able to detect immunoreactivity in dura, trigeminal (TG) and sphenopalatine ganglia (SPG) of

Ab181-dosed rats, but not in rats dosed with control antibody (Figure 4). In contrast, no labeling above background was detected in spinal trigeminal nucleus or superior salivary nucleus of the brainstem as well as in the hypothalamus and thalamus indicating that Ab181 did not cross the blood-brain-barrier or the amount of antibodies that entered the CNS after intravenous injection was below the detection threshold of the immunohistochemical method (Figure 4).

Discussion

PACAP38, first described in 1989 [40], is one of the key neurotransmitters of the parasympathetic system. Soon after its discovery, preclinical evidence suggested effects beyond its parasympathetic role, in particular in specific pain pathways related to headache [56]. Neuroanatomical and functional data obtained since strongly suggests a prominent role in primary headaches including cluster headache and migraine. Our data may provide a plausible under-pinning biology for a potential clinical effect of PAC_1 blockade in these disorders.

First, PACAP receptors (PAC_1, $VPAC_1$ and $VPAC_2$) are located in several key areas along the trigeminovascular system. Within the central part of the pain pathways processing trigeminal pain they have been identified in the thalamus [28], hypothalamus [28] and the TCC [35] while in the peripheral part, they are located in the TG [35] and the meningeal vasculature [5,6]. Beyond that, they are found in the SPG [8] highlighting the relevance of PACAP38 in the parasympathetic system, as well as its potential role in the functional interaction between the trigeminal and parasympathetic systems, namely the trigeminoautonomic reflex.

Secondly, from a functional perspective, PACAP38 may play a role in cluster headache and migraine since its plasma concentration is elevated in spontaneous attacks [52,55] and normalizes after effective attack-abortion with sumatriptan [55]. In addition, PACAP38 triggers migraine-like attacks in migraineurs [26,44], most likely through an activation of the PAC_1 receptor [55]. In the context of migraine, the ability to trigger attacks clearly distinguishes PACAP38 from VIP. These clinical observations can now be explored in specific

animal models. For example, electrical stimulation of the superior sagittal sinus of the cat induces the release of PACAP38 into the cranial circulation [55,56]. In line, the administration of PACAP38 facilitates nociceptive neuronal activity in the TCC [1]. These functional features of PACAP38 strikingly resemble the preclinical [50] and clinical observations [16,17] made with CGRP which predicted clinical efficacy of six small molecule CGRP receptor antagonists [10,29,31,38,42,54] and CGRP/CGRP-receptor antibodies [11,23,46,48] in the acute and preventive treatment of migraine as well as a CGRP antibody in the preventive treatment of episodic cluster headache [13].

Thirdly, the parasympathetic and trigeminal sensory systems connect and interact with each other. From an anatomic perspective, CGRP-positive sensory fibers project from the TG to the SPG [8] and *in vivo* data demonstrates that PACAP38 induces the release of CGRP from the TCC [35]. Nevertheless, the detailed molecular mechanisms behind the attack-triggering capability of PACAP38 and in particular the clinical relevance of the above described mechanism remain to be fully elucidated. Interestingly, the administration of PACAP38, despite creating an activation of the trigeminovascular system with the clinical picture of a migraine-like attack, does not induce the release of CGRP in a human model of migraine [26]. These results suggest that the PACAP38-induced effects on trigeminal activation are likely to be the result of a direct effect on nociceptive trigeminal neurons rather than a CGRP-mediated mechanism although both mechanisms do not exclude each other. In the case of a direct activation of either of the PACAP receptors, PACAP38 stimulates the cAMP/PKA pathway thereby increasing neuronal excitability [49]. In addition, all PACAP receptors may be modulated in their expression profile under chronic pain conditions [58]. It may be speculated that these up- and down regulating properties and the ability to

produce neuronal sensitization may even play a role in the chronification of migraine. Based on this large body of evidence, we set out to develop a specific antibody against the PAC_1 receptor as this receptor, as outlined above, is most likely the most relevant PACAP receptor in mediating the attack-triggering effect of PACAP38 and to test its effects on a model system that represents significant elements of cluster headache and migraine pathophysiology [4].

PACAP38 is a non-selective agonist at all the PACAP receptors, and VIP is only mildly selective to the $VPAC_1$ and $VPAC_2$ receptors [27]. Maxadilan, on the other hand, is the most selective agonist at the PAC_1 receptor [41], which allowed us to measure pharmacodynamic activities specifically mediated through the PAC_1 receptor. Although some years have passed since PACAP38 was discovered [40], selective antagonists to the individual receptors in the PACAP receptor family are still lacking. Several peptide antagonists have been reported with mild selectivity between different receptors [5,9,25] but their pharmacokinetic properties, especially their very short plasma half-life, hindered their utility in *in vivo* pharmacology studies. Therefore, Ab181 was developed. As demonstrated in the Results section, it is a potent and selective antagonist at the PAC_1 receptor, with no activity at the VPAC receptors. It also potent and selective inhibitor of maxadilan-induced increases in dermal blood flow in a time- and dose-dependent manner. Through the *in vitro* and *in vivo* profiling, Ab181 demonstrated full target coverage of the PAC_1 receptor at 3.5 h after intravenous administration and maintained sustained plasma concentration throughout the study duration. It is therefore the first ideal tool for the studying of selective PAC_1 pharmacology in preclinical species.

The new results of the study show that the intravenous administration of Ab181 inhibits stimulus-evoked nociceptive activity in the TCC. Unlike conventional small molecular agents whose molecule weight (MW) are normally < 500, the much larger sized antibodies (MW > 145KD) generally have a slower tissue distribution rate. As a result, it requires a substantial equilibrium period between the administration of the antibody and the initiation of the experiment before the assessment of its influence on stimulus-evoked activity. Therefore, the experimental design resembles more of a short-term preventive scenario than an acute reversal of nociceptive trigeminal activation. The results show that the extent of neuronal inhibition was almost identical to that observed with sumatriptan, suggesting a robust effect. The fact that we did not observe an effect on non-specific background activity reflects the specificity of the effect on nociceptive neuronal transmission.

~~and, beyond that, may also suggest a purely (or mainly) peripheral site of action.~~

The exact site of action of PAC_1 remains speculative. Literature suggested that agonist PACAP38, with MW >4000, triggers migraine-like attacks without crossing the blood-brain-barrier [12]. However, passage of PACAP27 and PACAP38 across the blood-brain barrier has been suggested [3]. Here, immunohistochemical analysis of Ab181 found a minimum presence in CNS which suggests that the site of the modulating action on nociceptive activity is likely to be located outside of the CNS. This would be in line with the results obtained from the electrophysiological experiments showing no effect on neuronal background activity as well as the immunohistochemical analysis in which Ab181 was detected in the relevant peripheral structures but not within the CNS. These findings suggest that Ab181 does not cross the blood-brain barrier in a significant concentration, a finding that is expected given the molecular size of the antibody. It cannot be excluded that

a small amount of antibody reaches the CNS but remains below the immunohistochemical detection limit, however, target coverage with such small amount is unlikely sufficient for a full antagonism effect. Therefore, the current study with a selective PAC_1 antagonist antibody supports the hypothesis that peripheral PAC_1 receptor inhibition can be sufficient to abort or prevent attacks of cluster headache and migraine.

Recently, a randomized, placebo-controlled trial (Phase 2) with a monoclonal PAC_1 receptor antibody (AMG 301) was reported in abstract form to be ineffective in the preventive treatment of migraine. In the context of our data some comments are relevant. First, in our experiments we used an antibody that was developed for rodents and differs significantly in its pharmacological properties, including its affinity to the PAC_1 receptor, for which it is more potent than the one used in the human Phase 2 trial. ~~from the antibody used in the human Phase 2 trial.~~ Secondly, while the available evidence suggests that the PAC_1 receptor plays a relevant role in trigeminal activation, the possibility that VPAC receptors are clinically significant is an open issue. The fact that VIP may trigger migraine attacks in subset of migraineurs may support this hypothesis [2]. Finally, in the clinical trial patients were not stratified by the presence or absence of cranial autonomic symptoms. Therefore, it may be hypothesized that the ability of PACAP38 to induce migraine attacks may require an action on PAC_1 receptors on trigeminal and parasympathetic neurons thereby increasing the activation of the trigeminoautonomic reflex. If this would be the case one could speculate of a higher relevance of this potentially therapeutic mechanism in migraine with cranial autonomic symptoms or in cluster headache.

Taken together, the findings of our studies suggest that the PAC_1 antibody Ab181 can inhibit nociceptive neuronal traffic so that this pharmacological approach may offer a new strategy for the preventive treatment of primary headache disorders, including cluster headache and migraine.

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Conflicts of interest

Jan Hoffmann is consulting for and/or serves on advisory boards of Allergan, Autonomic Technologies Inc. (ATI), Chordate Medical AB, Eli Lilly, Hormosan Pharma, Novartis and Teva. He has received honoraria for speaking from Allergan, Chordate Medical AB, Novartis and Teva. He received personal fees for MedicoLegal Work and from Quintessence Publishing.

Silke Miller is an employee of Amgen Inc.

Margarida Martins-Oliveira does not report any conflict of interest.

Simon Akerman reports an unrestricted grant, honoraria and travel reimbursements from electroCore LLC, unrelated to the submitted work.

Weera Supronsinchai does not report any conflict of interest.

Hong Sun is an employee of Amgen Inc.

Licheng Shi is an employee of Amgen Inc.

Dawn Zhu is an employee of Amgen Inc.

Sonya Lehto is an employee of Amgen Inc.

Hantao Liu is an employee of Amgen Inc.

Ruoyuan Yin is an employee of Amgen Inc.

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Figures

<u>Fig. 1:</u> Antagonist activity of Ab181 at rat rPAC1 CHO cell (rPAC1-CHO) against PACAP38 or maxadilan

The antagonist activity of Ab181 at the rat PAC_1 receptor was carried out by measuring the potency of Ab181 in inhibiting EC_{80} of agonist PACAP38 or maxadilan stimulated cAMP production. PACAP6-38, a PAC_1 receptor antagonist, was used as a positive control.

Full antagonist activity of Ab181 against both PACAP38 (A) and maxadilan (B) induced cAMP accumulation was observed in rPAC1-CHO cells with IC_{50} of 20 ± 3.3 nM (n = 3) and 4.5 ± 0.1 nM (n = 2) against respective agonist.

<u>Fig 2.1:</u> Prophylactic effect of Ab181 on maxadilan-induced increase in DBF in rats 48 h post drug treatment

Rats were given i.v. bolus injection of Ab181 (0.1, 0.3, 1 and 10 mg/kg, n = 6-7 rats/group). 48 h later, after obtaining a post-drug baseline (BL), maxadilan at a dose of 10 ng (in 20 µl DPBS) was injected intradermally. The dermal blood flow (DBF) was measured by a laser Doppler imager. Graph A: The increase in DBF (change from BL) caused by maxadilan injection over 60 min was expressed as % change from BL [= 100 x (individual post-maxadilan flux - individual BL flux)/individual BL flux]. Graph B: % change from BL (left-axis) versus resulting serum concentrations (right-axis) at 15 min post maxadilan; Solid triangle represents % change from BL at 48 h post drug treatment (left-axis, mean ± SEM), open bar represents serum concentration at 48 h post drug treatment (right-axis, mean ± SD). ***p<0.001, ****p<0.0001 compared to vehicle-treated group by one-way ANOVA followed by Dunnett's MCT.

Fig 2.2: Effect of Ab181 on a maxadilan-induced increase in DBF in rats 3.25 h post treatment

Rats were given i.v. bolus injection of Ab181 (0.1, 0.3, 1, 3 and 10 mg/kg, n = 7-8 rats/group). 3 h later, after obtaining a post-drug baseline (BL), maxadilan at a dose of 10 ng (in 20 µl DPBS) was injected intradermally. The dermal blood flow was measured by a laser Doppler imager. Solid triangle represents % change from baseline (mean ± SEM, left axis), open bar represents resulting serum concentration (mean ± SD, right axis). Control: Dummy antibody + vehicle (n = 13 rats). **p<0.01, ****p<0.0001 compared to control group by one-way ANOVA followed by Dunnett's MCT.

Fig 2.3: Time-course of effect of Ab181 (at 10 mg/kg) on a maxadilan-induced increase in DBF in rats

Rats were given i.v. bolus injection of Ab181 at 10 mg/kg with pretreatment time at 0.5, 3, 6.25, 48 and 168 h prior to maxadilan challenge (n = 3-8 rats/group). After obtaining a post-drug baseline (BL), maxadilan at a dose of 10 ng (in 20 µl DPBS) was injected intradermally. The dermal blood flow was measured by a laser Doppler imager. Solid triangle represents % Inhibition (mean ± SEM, left-axis), open bar represents resulting serum concentration (mean ± SD, right-axis).

Figure 3.1: Timeline of the electrophysiological *in vivo* experiments

Following the surgical procedure and the recording of baseline activity either Ab181 (10 mg/kg) or vehicle (A5Su) were injected intravenously. After a resting period of 2.5 h to allow the distribution of the antibody throughout the circulation and the relevant tissues, sumatriptan or its vehicle were administered intravenously. Following another resting

period of 30 min to allow sumatriptan to act, stimulus-evoked and spontaneous neuronal activity was recorded in the TCC.

Figure 3.2: Effect of Ab181 on neuronal activity in the TCC

Ab181 effectively inhibited stimulus-evoked neuronal activity in the TCC (A). The maximum inhibition was very similar to the one observed after the intravenous administration of sumatriptan (B). In contrast, an inhibiting effect on spontaneous background activity was not observed (C). Ab181 did not affect arterial blood pressure throughout the entire course of the experiment (D).

Figure 4: Distribution of Ab181 in tissues after intravenous dosing

Ex vivo detection of the IgG-portion of Ab181 or control antibody after i.v. dosing in dural vessels, trigeminal ganglion (TG) and sphenopalatine ganglion (SPG) as well as spinal trigeminal nucleus caudalis (TNC), superior salivatory nucleus (SSN) and thalamus. Staining of neuronal and glial structures is detected in dura, TG and SPG of Ab181-dosed rats, but not in TNC, SSN or thalamus. Only background staining is detected after intravenous dosing with control antibody (lower panels). Scale bars 50 µm.

Table 1: Antagonist Activities (IC_{50}) of Ab181

The antagonist activity of Ab181 at the rat PAC_1 receptor ($rPAC_1$) was carried out by measuring the potency of Ab181 in inhibiting EC_{80} of agonist PACAP38 or maxadilan stimulated cAMP production. PACAP6-38, a PAC_1 receptor antagonist, was used as a positive control.

Similar assay was used to determine the activity of Ab181 at rat $VPAC_1$ ($rVPAC_1$) and rat $VPAC_2$ ($rVPAC_2$) receptors against the EC_{80} of agonist VIP.

		$rPAC_1$		**$rVPAC_1$**	**$rVPAC_2$**
	Agonist	PACAP38	maxadilan	VIP	VIP
Ab181 (IC_{50}, nM)		20 ± 3.3 (n = 3)	4.5 ± 0.1 (n=2)	>1000 (n=2)	>1000 (n=2)
PACAP6-38 (IC_{50}, nM)		266 ± 77 (n = 3)	2.9 ± 1.5 (n=2)	430 ± 79 (n=2)	38 ± 4 (n=2)

IC_{50} values from these experiments (Table 1) are expressed as mean ± SD, and "n" represents the number of separate experiments.

Summary

PAC_1 receptor blockade using a monoclonal antibody inhibits nociceptive neuronal activity in the trigeminocervical complex.

Figure 1

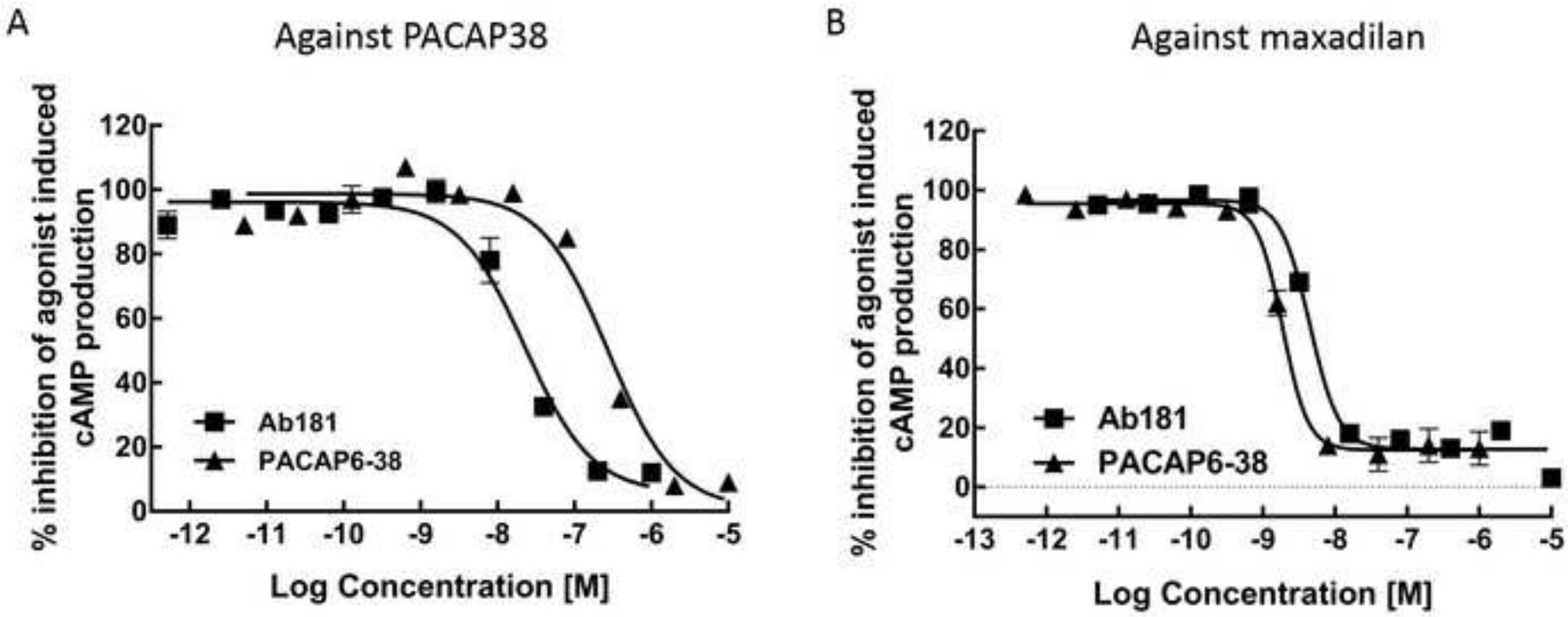
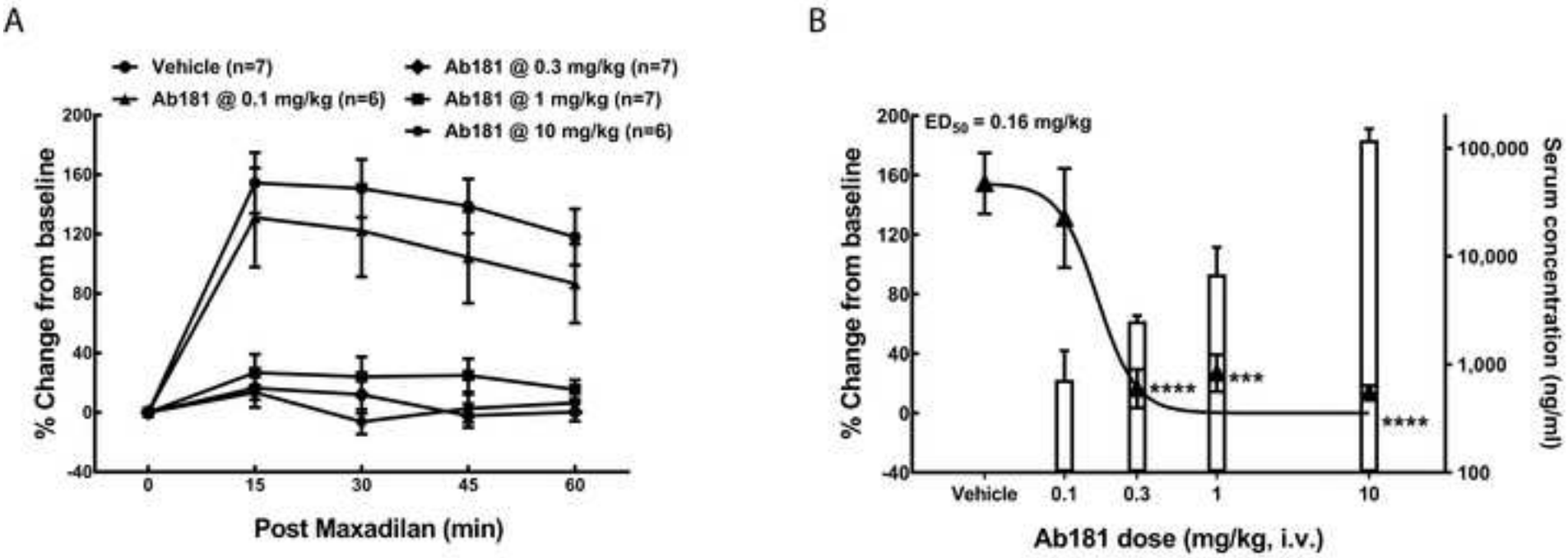


Figure 2.1



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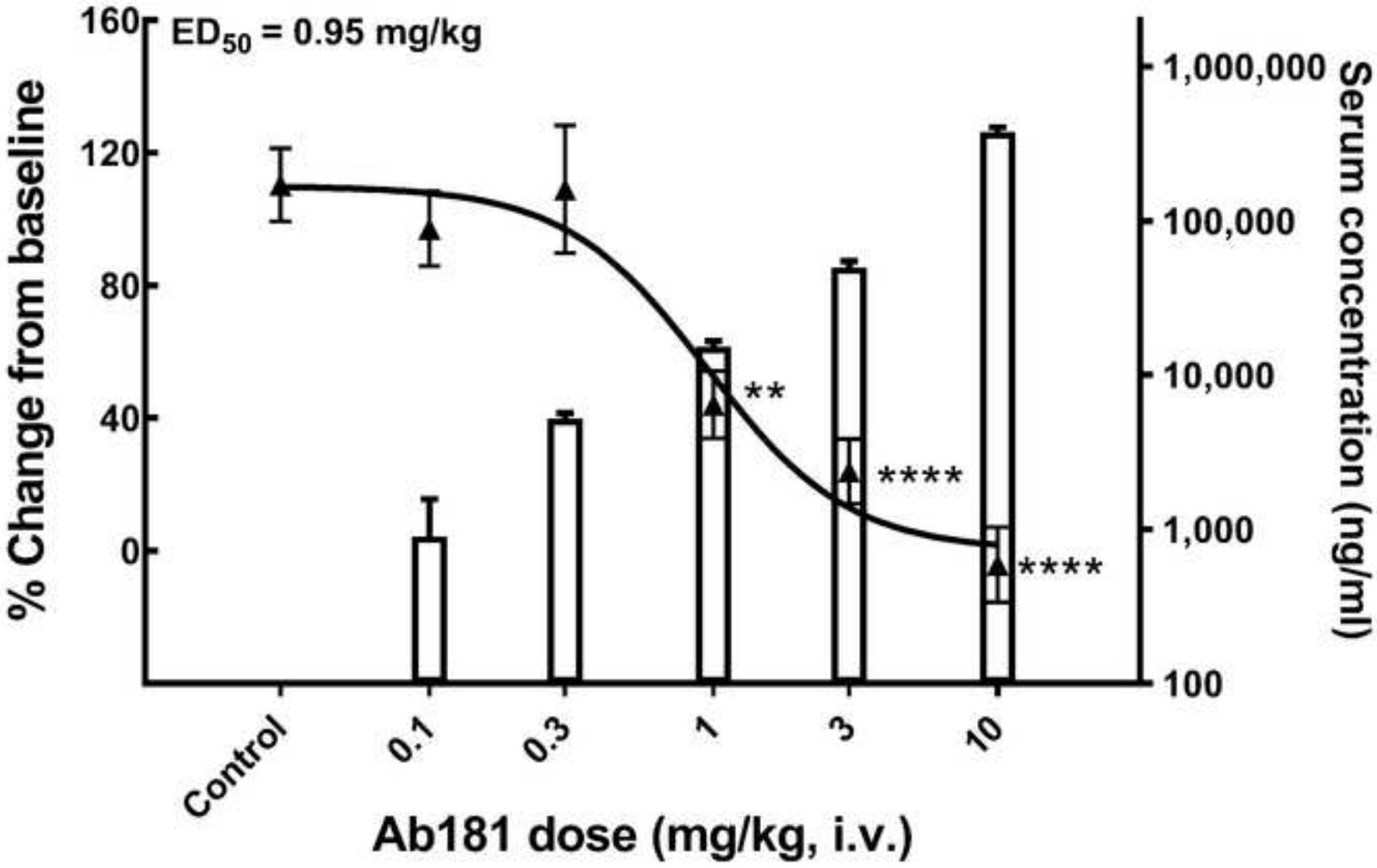

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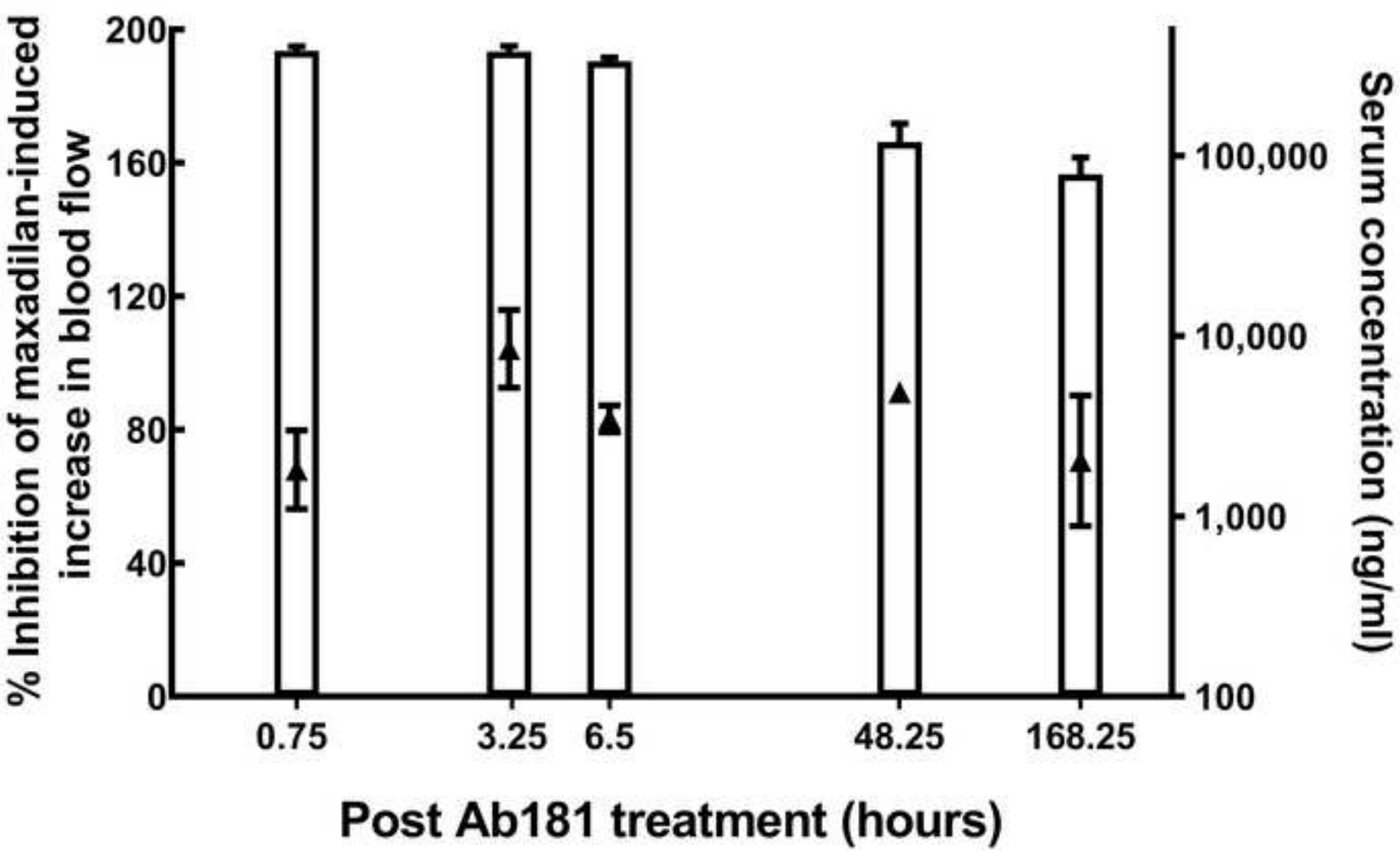

Figure 3.1

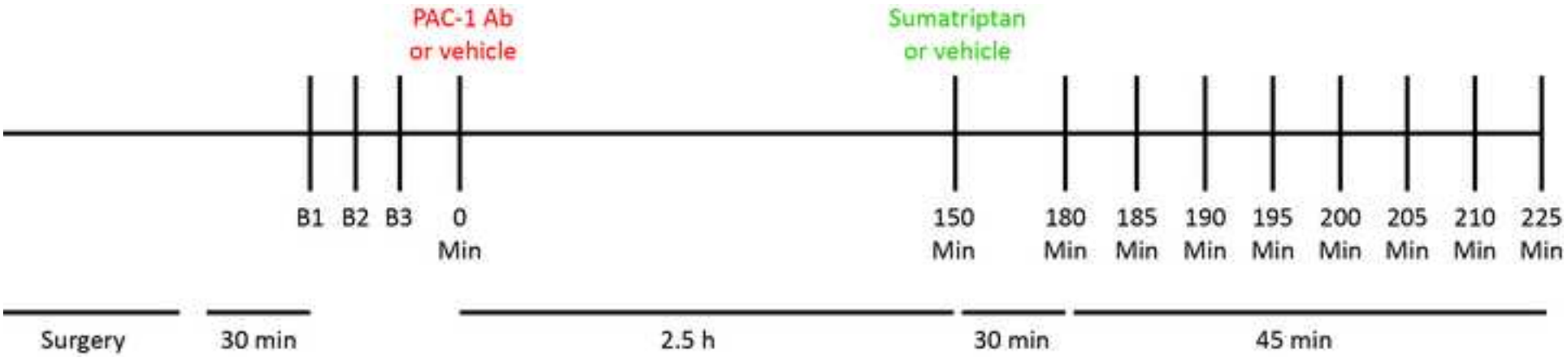

Figure 4

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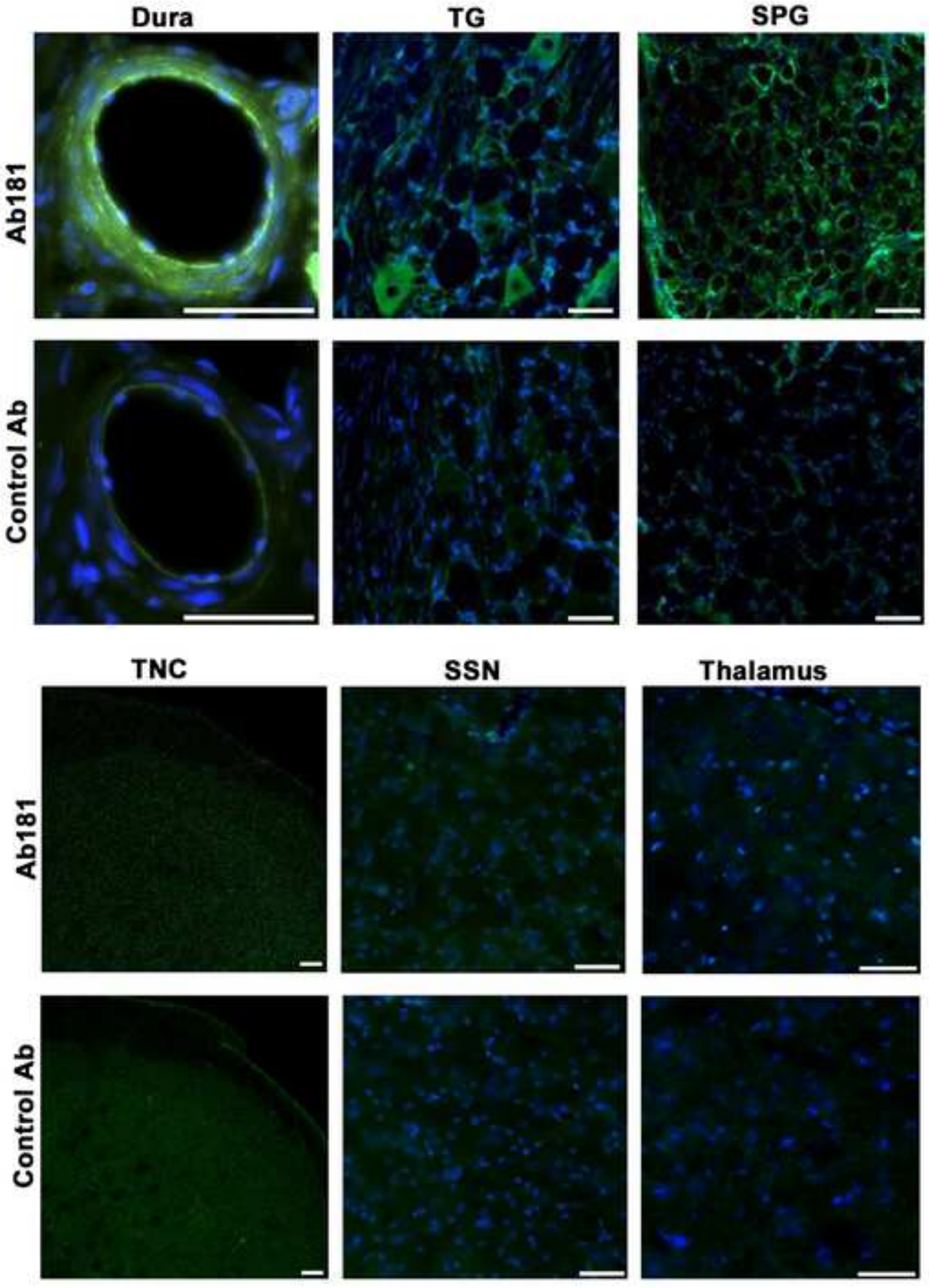

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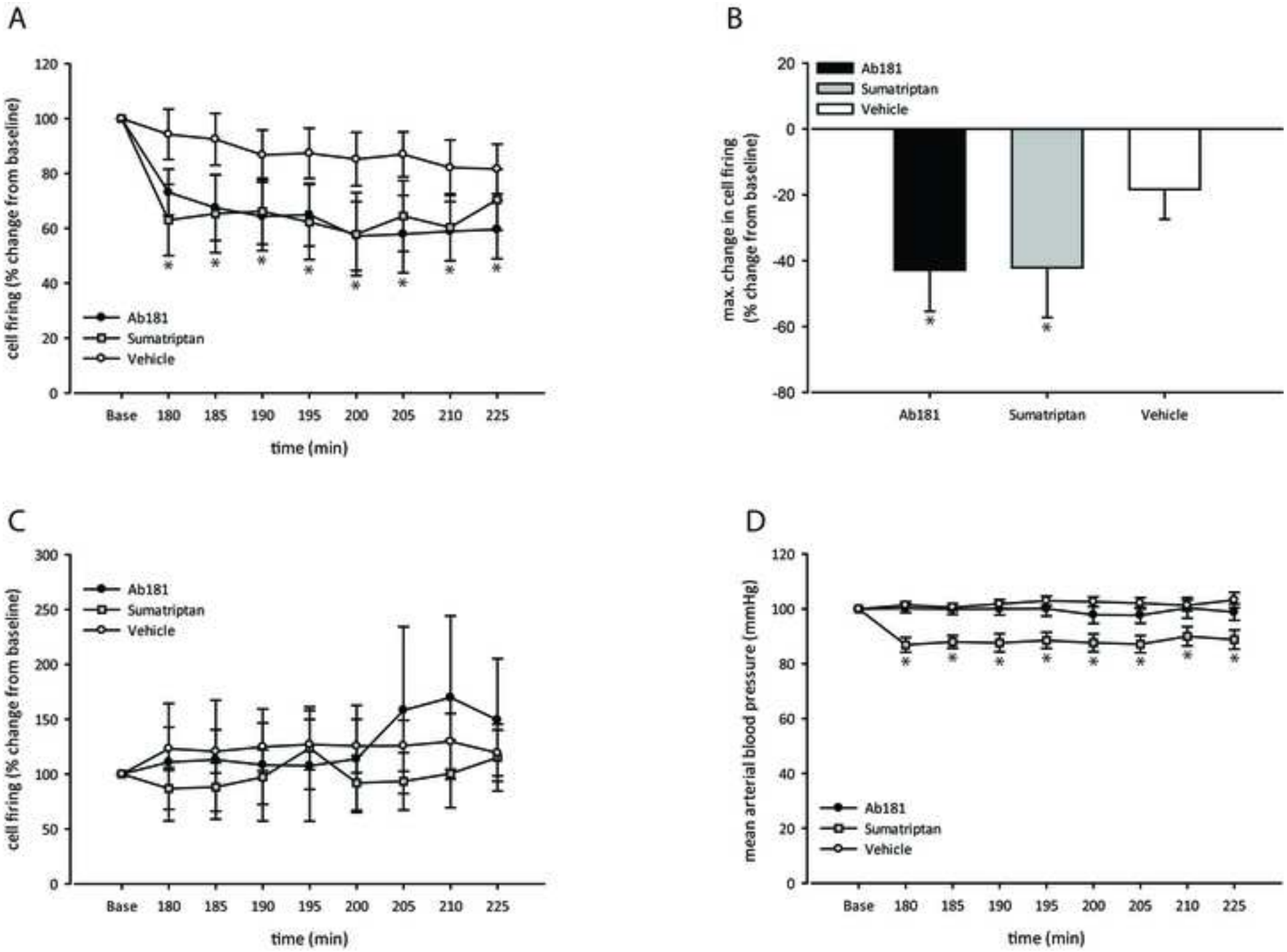

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c. Entire Agreement; Amendment. This Agreement sets forth the entire agreement of the parties on the subject hereof and supersedes all previous or contemporaneous oral or written representations or agreements relating to the rights and duties provided herein, and may not be modified or amended except by written agreement of the parties.

d. Force Majeure. Neither party shall be liable for any default or delay on its part in performing any obligation under this Agreement if such default or delay is caused by natural disaster, accident, war, civil disorder, strike or any other cause beyond the reasonable control of such party. In the event that either party is prevented by such an occurrence or circumstance for a period of more than ninety (90) days from fulfilling its obligations under this Agreement, the other party may terminate this Agreement upon thirty (30) days' written notice.

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f. Headings. All headings are for reference purposes only and shall not affect the meaning or interpretation of any provision hereof.

g. Severability. If any provision of this Agreement is held to be illegal, invalid, or unenforceable under the present or future laws, then such provision shall be revised by a court of competent jurisdiction to be enforceable if permitted under applicable law, and otherwise shall be fully severable. In any event, this Agreement shall be construed and enforced as if such illegal, invalid, or unenforceable provision had never comprised a part of this Agreement, and the remaining provisions of this Agreement shall remain in full force and effect and shall not be affected by the illegal, invalid, or unenforceable provision or by its severance from this Agreement.

h. Status of the Parties. The parties are independent contractors. Nothing in this Agreement is intended to or shall be construed to constitute or establish any agency, joint venture, partnership or fiduciary relationship between the parties, and neither party has the right or authority to bind the other party nor shall either party be responsible for the acts or omissions of the other.

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